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Wild Plants as Sources of the Permanency of Viruses Infecting Cultivated Plants: Case of Cassava Begomoviruses in Togo

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ABSTRACT

A study was carried out on some wild plants in order to understand the permanency of Cassava Begomoviruses diseases in Togo. Leaves were collected in cassava fields from cassava and some wild plants (*Albizia zygia* (DC) J.F. Mabr, *Senna hirsuta* (L.) H.S. Irvin and Barneby, *S. obtusifolia* (L.) H.S. Irvin and Barneby, *S. occidentalis* (L.) Link., *Manihot glaziovii* Müll. Arg., *Pupalia lappacea* (L.) Juss., *Strophanthus hispidus* DC) that exhibiting leaf curling, distorting and chlorotic lesions near the veins and stunting which were similar to the symptoms of Begomovirus infection and were analyzed by PCR assays with degenerate primers. Symptomatic leaf tissues from some of these infected plants were ground in 0.1 M phosphate buffer (1:1, w/v pH7.0) and squeezed through double layered muslin cloth and the filtrate was mechanically inoculated to several indicator species. Consistent amplification of DNA fragment was obtained (770 bp fragments) from symptomatic plants. The PCR analysis revealed the association of Cassava mosaic begomovirus in *Albizia zygia, Senna obtusifolia, Manihot glaziovii, Pupalia lappacea, Strophanthus hispidus*. The presence of the virus was confirmed in host inoculated plants. The presence of the virus was confirmed in inoculated host plants analysed by PCR.

Key words: Begomoviruses diseases, cassava, degenerate primers, PCR assays, wild plants

INTRODUCTION

Plant diseases are important constraints on worldwide crop production, accounting for losses of 10-30% of the global harvest each year (Strange and Scott, 2005). As a consequence, crop diseases represent a significant threat to ensuring global food security. To feed the growing human population it will be necessary to double food production by 2050, which will require the sustainable intensification of world agriculture in an era of unpredictable climate change (FAO, 2006; Evans *et al*., 2008). And with the persisting of the climate changes, insects which are most of the time, the vectors of agents of viral diseases of the cultures have left their various ecological niches bringing with them, agents of emergent viral diseases up till now unknown on vegetables and food

crops. Controlling the most important plant diseases represents one of the important means of delivering as much as possible, the current productivity of food crops. To accomplish this task, a fundamental understanding of the biology of plant infection by agents causing diseases, such as viruses, bacteria and fungi will be necessary (Strange and Scott, 2005). Concerning viruses, it is very important to understand the role of wild plants in the maintaining of these viruses in the environment. The aim of this study is to demonstrate that wild plants could be the key of the permanency of cassava mosaic diseases in cassava producing zones in Togo.

MATERIALS AND METHODS

Sources of virus: A survey was carried out in cassava production zones in order to evaluate the impact of Cassava Mosaic Disease (CMD) related to *Begomovirus*. During this investigation, period of 2004 and 2005, samples of cassava leaves and wild plants (*Albizia zygia*, *Senna hirsuta*, *S. obtusifolia*, *S. occidentalis*, *Manihot glaziovii*, *Pupalia lappacea*, *Strophanthus hispidus*) presenting symptoms of severe mosaic, yellowing and nanism, were collected in all the zones where cassava is produced in Togo. The different wild plants used in this study were identified by Dr. A.Y. Woegan of the Faculty of Sciences (University of Lomé-Togo).

Viral isolates of reference: The viral isolates of reference used in this study like positive controls, were provided by Dr. Peterschmitt of CIRAD-BIOS, Montpellier (France) and Dr. Max Schoenfelder of DSMZ (Germany). These characterized isolates utilized in this study are consigned in Table 1. Negative controls were also provided by the same laboratories.

Plant tests inoculation and PCR analysis: To confirm the presence of the ACMV and EACMV identified in *Albizia zygia*, *Senna obtusifolia*, *Manihot glaziovii*, *Pupalia lappacea*, *Strophanthus hispidus*, sap extracted from leaves of these infected plants was inoculated to healthy *Nicotiana benthamiana* seedlings. Thus, 100 mg fresh leaves of these infected plants were grounded in 1 mL (0.1 M phosphate buffer, pH 7.0) extraction buffer. Inoculum was rubbed onto the surface of the first two fully expanded *N. benthamiana* leaves with carborundum. Symptom development was recorded daily and the third leaf from the apex was removed and frozen every 5 days for PCR.

Extraction of the total DNA: The total viral DNA was extracted from the various foliar samples collected according to plant DNA mini preparation method of Dellaporta *et al.* (1983). Fifty milligram of cassava infected leaf tissue was ground in 500 µL of Dellaporta extraction buffer (100 mM Tris pH 8, 50 mM EDTA, 500 mM NaCl, 10 mM 2-mercaptoethanol) with pestle in 1.5 mL microtube and 33 µL of 20% (w/v) of SDS were added, the mix was vortexed and incubated at 65°C for 10 min. Then, 160 µL of 5 M potassium acetate were added to the extract and vortexed and finally centrifuged for 10 min at 14,000×g. The supernatant (580 µL) was transferred to new

Table 1: List of used positive controls

Reference number DSMZ	Origin	Description
PV-0421	Nigeria	ACMV-NG: Cassava infected leaves
PV-0423	Kenya	EACMV: Cassava infected leaves
PV-0872/Ca-161	Cameroun	EACMCV: Cassava infected leaves

Table 2: Specific primers used for the identification of ACMV, EACMV and ICMV

Primer	Sequence (5'-3')	Target region
JSP001	ATG TCG AAG CGA CCA GGA GAT	5' ACMV/EACMV CP
JSP002	TGT TTA TTA ATT GCC AAT ACT	3' ACMV CP
JSP003	CCT TTA TTA ATT TGT CAC TGC	3' EACMV CP

Fondong *et al*. (2000)

1.5 mL microtube and 350 µL of ice cold isopropanol was added, vortexed and centrifuged for 10 min at 4,000×g and the supernatant was removed and discarded; the pellet was dried for 5 min in a Speed-Vac dryer and resuspended in 50 µL of RNase A (10 µg mg^{-1} in 10 mM Tris-HCl pH 8, 1 mM EDTA) and left at room temperature for 30 min. DNA was finally precipitated for 30 min at -80°C in 2.5 volumes of absolute ethanol in the presence of 0.3 M sodium acetate. The pellet was washed twice in 1 mL of 70% ethanol, dried for 5 min in a Speed-Vac dryer and resuspended in 500 µL distilled water.

Diagnosis by PCR: Identification of the *Begomovirus* in the samples was done by Polymerase Chain Reaction (PCR) amplification using specific degenerate primers targeting the coat (CP), namely: JSP001/JSP002 and JSP001/JSP003 which identify respectively ACMV (*African cassava mosaic virus*) and EACMV (*East African cassava mosaic virus*) (Fondong *et al*., 2000). These primers are described in Table 2; JSP001/JSP002 and JSP001/JSP003 give fragments of 770 base pairs.

The negative and positive controls (Table 1) were provided by Dr. Max Schoenfelder of DSMZ (Germany). One of the positive controls for ACMV was provided by Dr. Peterschmitt of CIRAD (France) and was isolated in Côte d'Ivoire.

The PCR reaction was performed in a volume of 25 µL containing 2 µL of extracted DNA, 1 unit of Taq Polymerase Extrapol 1 (Eurobio), 1.5 mM $MgCl_2$, 100 µM of each dNTP and 0.8 µM of each primer in the reaction buffer provided by the manufacturer. The PCR conditions are as follows: A 94°C denaturation step of 2 min followed by 30 cycles of 30 sec at 94°C, 30 sec at annealing temperature (50°C denaturation for ACMV and EACMV) and 1 min at 72°C and then a final elongation step of 10 min at 72°C.

RESULTS

Three hundred and tweenty collected foliar samples from cassava leaves and wild plants (*Albizia zygia, Senna hirsuta, S. obtusifolia, S. occidentalis, Manihot glaziovii, Pupalia lappacea, Strophanthus hispidus*) presenting symptoms of severe mosaic, yellowing and nanism, were analyzed by PCR with degenerate primers specific to ACMV and EACMV. Among the analyzed samples, 262 were found to be infected by the two main cassava Begomoviruses (ACMV and EACMV). The analysis of the results revealed the presence of the two targeted Begomoviruses (Fig. 1a, b) in wild plants. The results also revealed that the two Begomoviruses (ACMV and EACMV) are often in mixed infection in the different regions. For instance, the proportion of samples infected by ACMV alone was 31.02%, that infected by EACMV alone was 10.10% and that in mixed infection was 40.56% (Table 3). It is important to notice that all the samples used in this study presented typical symptoms of Begomoviruses. About 18.32% of the analyzed samples were revealed to be negative to the primers used in this study.

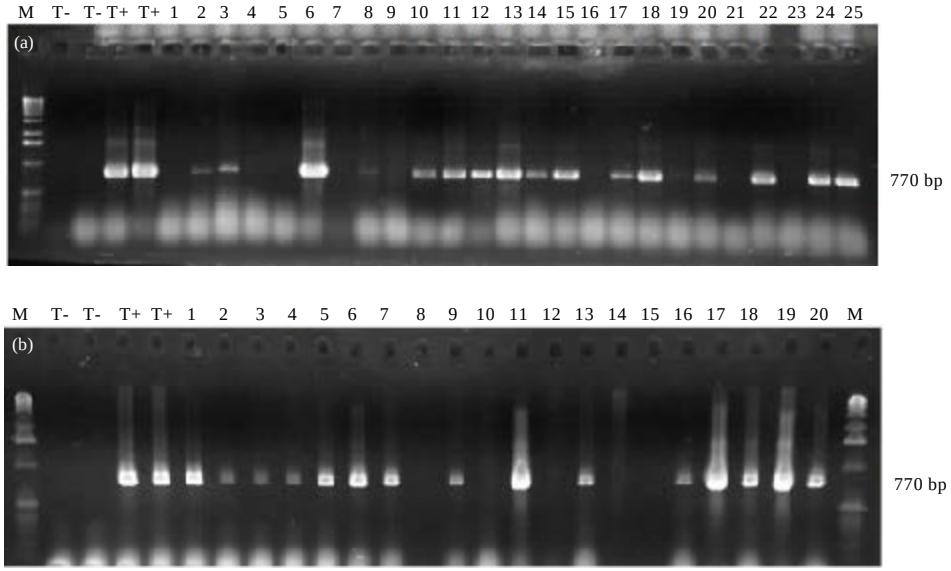

Fig. 1(a-b): Electrophoretogram showing the results obtained with the primer, (a) JSP001/JSP002 specific to ACMV giving fragments of 770 bp, 1-25: Isolates from different areas of cassava production in Togo, Samples 1, 4, 5, 7, 9, 16, 19, 21 and 23 of this electrophoretogram, reacted negatively to ACMV and (b) JSP001/JSP003 specific to EACMV giving fragments of 770 bp, 1-20: Isolates from different areas of cassava production in Togo. Samples 8, 10, 12, 14 and 15 of this electrophoretogram, reacted negatively to EACMV. M: Marker, T-: Negative control (healthy *N. benthamiana* (HNB)), T+: ACMV positive control (infected *N. benthamiana* (INB))

Table 3: Two begomovirus detected on samples analysed by PCR

		Samples with positive reactions					
Regions	Samples analyzed	ACMV alone	Percentage	EACMV alone	Percentage	ACMV+EACMV	Percentage
Central	85	26	30.59±3.24	9	10.59±1.12	34	40.00±4.24
Kara	65	20	30.77±7.61	4	6.15±1.52	26	40.00±9.90
Maritime	65	20	30.77±7.61	3	4.62±1.14	26	40.00±9.90
Plateaux	86	27	31.40±3.11	16	18.60±1.84	35	40.70±4.03
Savannas	19	6	31.58±18.09	2	10.53±1.06	8	42.11±2412
Total	320	99	31.02±7.93	34	10.10±2.33	129	40.56±1044

PCR diagnosis on wild plants: Among the samples collected on wild plants showing typical virus symptoms in cassava field in different regions and analyzed by PCR, samples from *Albizia zygia*, *Senna obtusifolia*, *Manihot glaziovii*, *Pupalia lappacea*, *Strophanthus hispidus* were found to be infected by ACMV and EACMV.

Reaction in *N. benthamiana* and PCR analysis: Healthy *Nicotiana benthamiana* seedlings inoculated with sap extracted from infected leaves of *Albizia zygia*, *Senna obtusifolia*, *Manihot glaziovii*, *Pupalia lappacea* and *Strophanthus hispidus* (Fig. 1a, b) and analyzed by PCR, confirmed the occurrence of ACMV and EACMV in these wild plants in Togo. It is important to

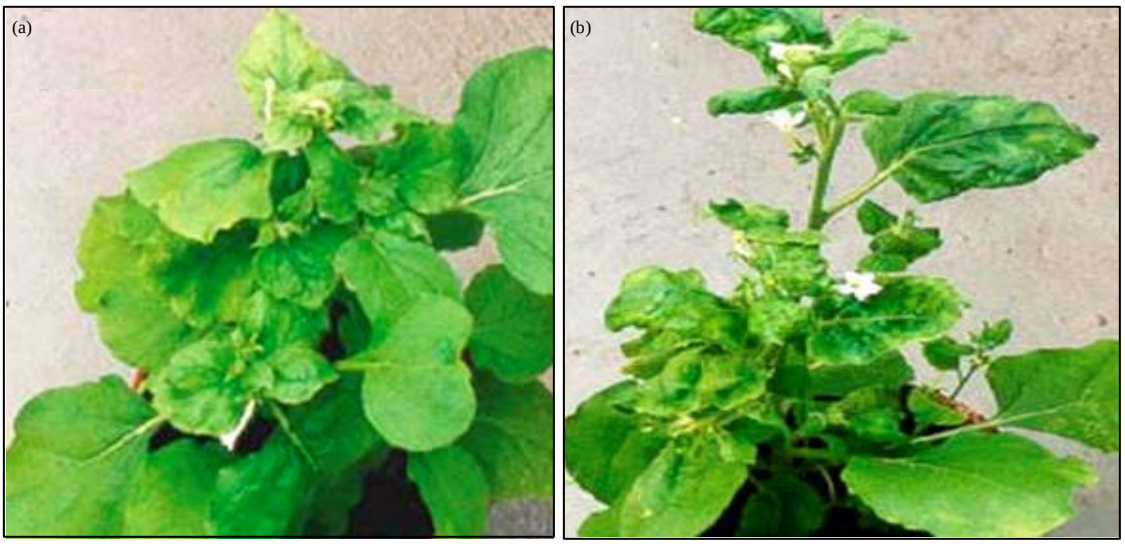

Fig. 2(a-b): Reaction of inoculation of *N. benthamiana* to *N. benthamiana* initially infected by sap from infected *Strophanthus hispidus*, (a) *N. benthamiana* initially infected by ACMV and (b) *N. benthamiana* initially infected by EACMV

notice that for sap extracted from wild plants infected by ACMV and EACMV and inoculated to healthy *Nicotiana benthamiana* seedlings, symptoms were not characteristics of these two viruses, regardless of the inoculums concentration employed. But the reinoculation from infected *N. benthamiana* by sap from these infected wild plants to *N. benthamiana*, the symptoms were characteristic of these two viruses (Fig. 2). The PCR analysis using the different primers used in this study proved the presence of the respective viruses identified on systemically affected leaves of all inoculated plants at 10 days post-inoculation for ACMV and approximately 30 days post-inoculation for EACMV.

DISCUSSION

From this study, it is very clear that wild plants play an important role in the maintenance of these Begomoviruses in the environment and thus as sources of the permanency of these virus agents infecting cultivated plants essentially cassava. Of the randomly analyzed samples (320 samples in total), two hundred and sixty two of all the tested samples (from wild and cassava plants) were positive in PCR amplification using Begomoviruses specific primers. All of the positive samples were infected with (ACMV and EACMV) and most of them (41%) showed mixed infection. As it was demonstrated by Padidam *et al.* (1999), Begomoviruses are known to be fluent to recombination and if this assertion is true, that means that new Begomoviruses could be now in the environment implying new recombination and creating new Begomoviruses and this could explain the 18.32% of the samples which did not give positive reaction to the primers used in his study. So, evidence is provided here of the relevance of the wild species (*Albizia zygia*, *Senna obtusifolia*, *Manihot glaziovii*, *Pupalia lappacea* and *Strophanthus hispidus*) as reservoir of Begomoviruses that causes epidemics of CMD and of recombination as a force driving their evolution. Moreover, co-infections were shown to be common (Table 3) and the presence of a previously unreported Begomovirus variant (UgV/Tg) was demonstrated (Adjata *et al.*, 2009). Interestingly, this variant

exhibited a recombinant nature and novel pathogenic properties that can result in enhanced long-term ecological fitness. In Togo, diseases caused by whitefly-transmitted Begomoviruses are on increase, affecting the cultivation and economic yield of important plants. It is important to notice that the identification of Begomoviruses on these wild plants is not specific to a particular cassava production zone; what is obvious now, is that in the absence of whiteflies' plants of predilection, these whiteflies would have the habit to use intermediate hosts to survive and to wait for their hosts which are often important economic cultivated plants.

The coexistence of these two Begomoviruses which often infect cassava in mixed infections, could explain the severity of the symptoms observed in the main zones of cassava production in Togo. But it is also now known that out of synergism phenomenon, the severity of a symptom on a plant could depend on many other factors; severe strains resulting from recombination phenomenon could explain symptom severity on infected plants. Increased overlapping cultivation of cassava and closely related plants has certainly resulted in an expanded host range of existing Begomoviruses and has led to the emergence of new strains and recombinants like Uganda variant in Togo (Adjata *et al.*, 2009). In other hand, this study highlights a situation which was never considered in previous studies; the fact that plant belonging to other botanic families could be cassava Begomovirus source of inoculums. That is to say, viruses should have overcome species barrier.

This is especially true for cassava, which is grown in diverse agroclimatic zones throughout the year, thus perpetuating the virus inoculums and vector population (Varma *et al.*, 2011). In this context, considerable progress has been made in characterizing cassava Begomoviruses in cassava production zones in Togo, but many remain to do.

The inoculation of sap extracted from infected leaves of *Albizia zygia*, *Senna obtusifolia*, *Manihot glaziovii*, *Pupalia lappacea* and *Strophanthus hispidus* to infect healthy *Nicotiana benthamiana* seedlings revealed that these wild plants could play an important role in the maintenance of the agents of the disease in the environment which is a serious problem to crop production (Varma and Malathi, 2003). The result obtained from these inoculations showed surprisingly that not only sap extracted from wild plants infected by ACMV and EACMV, could infect *N. benthamiana*, regardless of the inoculums concentration employed, but also, the two *Begomoviruses* (ACMV and EACMV) could be transmitted through these infected wild plants. This observation is very important because in open environment, the vectors could come from any source of inoculums no matter the species.

CONCLUSION

The important result of this study was that wild plant could play an important role in the maintenance and the spread of agents causing cassava mosaic disease in cassava production zones in Togo. And this is very imperative to be taken into account because producers in Togo are not under greenhouses; they work in an open environment where they cannot control the main vector, *B. tabaci*, of these cassava Begomoviruses. Through this study, it is now known that whiteflies would have many other intermediate hosts until now unknown. Thus, selection programmers should take into account this situation linked to climate changes.

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